FORMATION OF A FAT PROTEIN COMPLEX IN MILK BY HOMOGENIZATION

K. K. FOX, VIRGINIA H. HOLSINGER, JEANNE CAHA, AND M. J. PALLANSCH
Dairy Products Laboratory, Eastern Utilization Research and Development Division, USDA
Washington, D. C.

SUMMARY

A fat-protein complex which sediments in a centrifugal field is produced in milk by homogenization. Fat-protein complex formation is found to increase with increasing homogenizing pressure; with increasing fat content; with an increase in concentration above 31% total solids (T.S.); and with increasing calcium concentration up to a limit of 10 m*M* added calcium. In a suspending medium of increased density produced by adding sucrose, the fat-protein complex formed by homogenizing concentrated milk of more than 31% T.S. is found to consist of a single entity. In nonconcentrated milk, the fat-protein complexes have a range of fat-nitrogen ratios.

Experiments to determine the manner in which the fat-protein complex is formed indicate that conditions existing at the homogenizer valve at the instant of homogenizing are responsible for complex formation. Ultracentrifuge analyses of the protein moiety of the fat-protein complex show it to be casein.

Currently, in the Dairy Products Laboratory of the Eastern Utilization Research and Development Division, the unique physical properties of foam-dried whole milk powder (7) are being investigated. Upon centrifuging a reconstituted sample of this powder, fat was found associated with the casein in the sediment. Investigation showed that a fat-protein complex was formed during the homogenization step preceding drying. Since the amount of protein-fat complex would conceivably account for some of the physical properties of foam-dried powder, fat-protein complex formation was subjected to further research.

The proteins normally adsorbed on the fat globules have been the subject of much study (6). Brunner, Duncan, and Trout (2) found that the proteins bound on the fat globules of homogenized milk were different from the proteins in nonhomogenized milk. In these earlier studies attention was given to the protein-fat complex which floated in milk subjected to centrifugation. This paper, in contrast, is concerned only with those fat-protein interaction products of sufficient density to sediment in milk subjected to high centrifugal fields.

This paper describes formation of sedimentable fat-protein complexes in terms of: (1) homogenization pressure, (2) total solids concentration, and (3) fat to solids-not-fat ratios. Experiments designed to discern the mechanism responsible for the fat-protein interaction also are described.

EXPERIMENTAL PROCEDURE

Material. All milk used in this study was obtained from mixed herds located at the Beltsville Experiment Station of the U. S. Department of Agriculture.

The raw milk was routinely standardized and pasteurized at 145° F. for

30 min. Concentrates were prepared from the pasteurized milk by using a single-effect Rogers Pan.[1]

Homogenization. Milk and milk concentrates were homogenized at 145° F. using a single stage of a Manton Gaulin Model 75 homogenizer [1] equipped with a Dyna Jet Homogenizing valve.[1]

Reconstitution. After homogenizing, the concentrates were diluted with distilled water to the solids content of the unconcentrated milk.. The reconstituted and nonconcentrated milks were both held at 36° F. for 48 hr. to allow the internal milk systems to come to equilibrium. This holding period was necessary to secure reproducibility of quantitative measurements. The samples were warmed to 104° F. to put the fat in the liquid state, and centrifuged at this temperature.

The samples were centrifuged in a Spinco Model L[1] preparatory centrifuge using a No. 40 rotor. They were centrifuged at 150,000 × G (av.) for 90 min. Nitrogen depletion-rate measurements on nonhomogenized milk indicated that this centrifugal force was almost six times greater than necessary to sediment the caseinate complex.

Nitrogen determinations were made by the micro-Kjeldahl method of the A.O.A.C. (1). Fat analyses were made by the Mojonnier method.

For examining the protein of the fat-protein-fat complex, a Spinco Model E[1] ultracentrifuge and an Aminco[1] electrophoresis apparatus were used. The protein was suspended in a veronal buffer, pH 8.4, total ionic strength 0.1, containing 0.05 *M* NaCl.

Analytical technique. After centrifuging, a pellicle of fat was found at the top of the centrifuge tube, the amount depending upon the homogenizing pressure. This fat was discarded. The supernatant liquid was collected and analyzed for nitrogen. The sediment was recovered and analyzed for fat and nitrogen. The sediment was always in the form of a firm pellet in the bottom of the centrifuge tube. These pellets were ground in a mortar and weighed samples taken for fat and moisture determinations. It was found that the wet sediment could be easily dissolved for fat analysis by adding 10 ml. of ethylenediaminetetracetic acid (E.D.T.A.) solution (eq. to 0.6 mg. Ca^{++} ml.) to the Mojonnier[1] flasks containing the weighed sample (1-2 g.). The moisture content was found by drying the wet sediment in a vacuum oven for 12 hr. at 105° C. An aliquot of the dried sediment was taken for nitrogen analysis. Fat analysis by the Mojonnier method could not be made on the dried sediment.

RESULTS

Extent of complex formation. The effect of homogenization pressure on formation of a sedimentable fat-protein complex is shown in Figure 1. The milk contained 12.4% T.S., of which 3.3% was fat. The different concentrations are indicated as 1:1 (unconcentrated), 2.5:1 (31% T.S.), 3:1 (37.2% T.S.), and 4:1 (49.6% T.S.). At all concentrations the ratio of T.S. to fat is the

[1] It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

same. The graphs represent the average of three determinations. The values for a 2:1 concentration are shown in Figure 2. Likewise in Figure 1, data for homogenizing pressures of 1,000 and 6,000 p.s.i. are shown but are not included in Figure 2.

Figure 1 shows that increasing the homogenizing pressure causes an increase in the formation of a sedimentable fat-protein complex at all concentrations. It is also seen that the homogenizing pressure range normally used in industry produces the fat-protein complex.

In Figure 2, the fraction of the fat sedimenting is plotted versus the T.S. concentration for three of the homogenizing pressures studied. It is observed that above a concentration of 31% T.S. at the fat level used, a significant increase in the amount of fat bound to the protein occurs. Because of the deviations in the data at 37% T.S. this concentration is considered to be at or near the critical point for the occurrence of some factor responsible for the increased binding of the fat to the protein. More information regarding this factor is presented later.

Identity of the protein in the complex. The identity of the protein involved in the complex is established in part or wholly by several methods.

a. Amount of protein required to sediment the fat. By reference to Figures 1 or 2 it can be seen that when a 4:1 (49.6% T.S.) concentrated milk is homogenized at 8,000 p.s.i. about 75% of the fat sediments. Since the milk

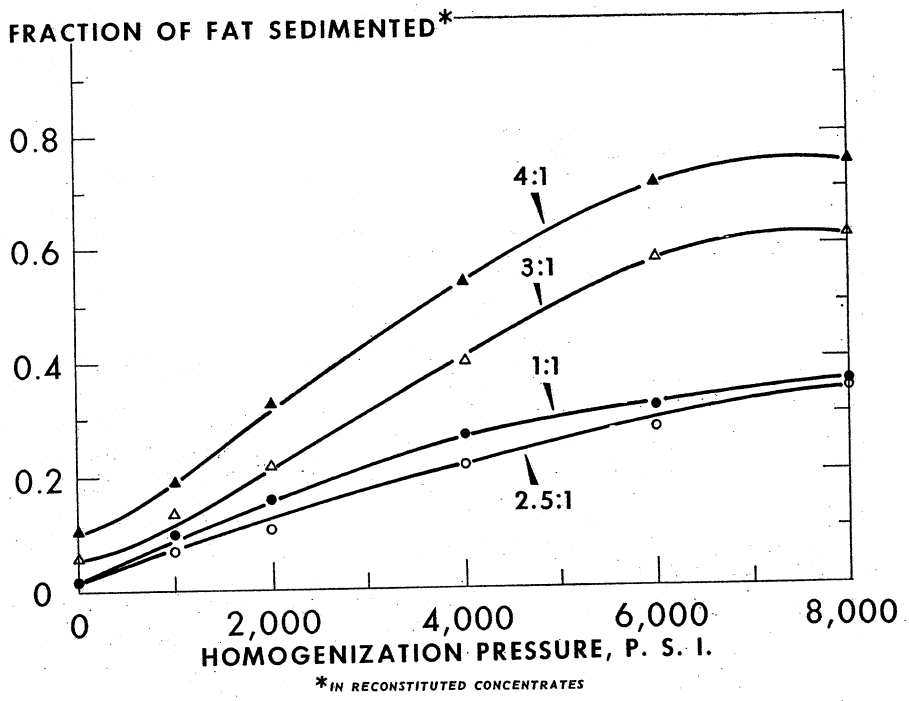

FIG. 1. Effect of homogenization pressure on fat-protein complex formation in milk and concentrated milks.

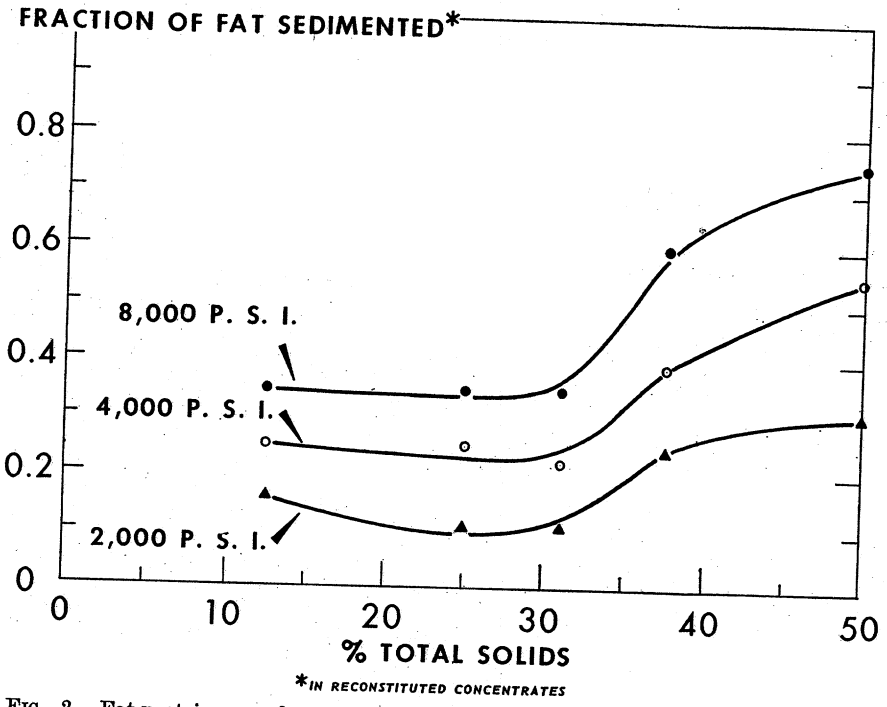

FIG. 2. Fat-protein complex formation as a function of concentration at different homogenization pressures.

contains 3.3% fat, about 2.5 g. (75%) of fat is sedimented from a 100-g. sample. The density of the supernatant was 1.026 g/ml. This would be a limiting minimum value for the density of the complex, i.e., with this density it would remain suspended rather than sediment. Assuming the density of milk fat to be 0.93 g/ml, and using a value of 0.72 as the apparent specific volume of the protein, 0.78 g. is the calculated amount of protein required to produce a complex of density 1.026 g/ml. If the milk contained 3.5 g. protein of which 80% was casein, the 0.78 g. of protein is slightly greater than the amount of whey proteins present. Since the complex readily sediments instead of remaining suspended, and because higher fat to protein ratios have been observed (see Figure 4), casein must be a part of the complex.

b. The fat is removed by resuspending the complex in a solution of E.D.T.A., but is not removed in warm water. Also, the calcium-nitrogen ratio of the fat-protein complex is the same as that of the calcium caseinate complex (4). Both of these facts imply that the calcium caseinate complex is involved in the fat-protein interaction.

c. Ultracentrifuge data. To establish the identity of the protein in the sedimenting fat-protein complex, the complex was sedimented from a reconstituted 4:1 concentrated milk which had been homogenized at 8,000 p.s.i. It was washed two times by dispersing it in distilled water with a Waring Blendor. The washed complex was then dispersed in distilled water by blending, and

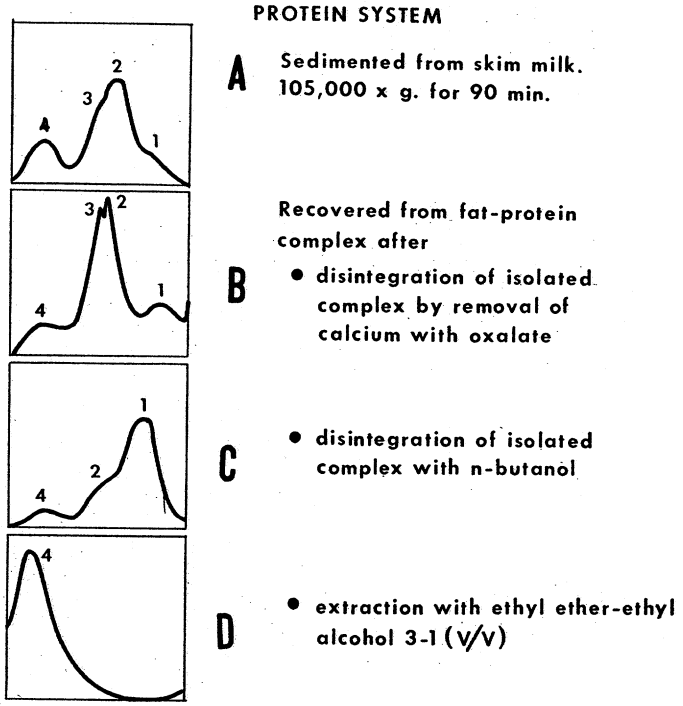

FIG. 3. Ultracentrifuge patterns of protein involved in complex 1, 2.

sufficient sucrose added to raise the specific gravity to 1.09. The solution was centrifuged and the fat-protein complex recovered by flotation. The flotated complex was dispersed in distilled water and washed two times with distilled water. The isolated complex had a fat/nitrogen (F/N) ratio of 9.1:1 and a calcium/nitrogen (Ca/N) ratio of 0.216. The protein entity was separated from the complex by several methods noted below, and its ultracentrifugal and electrophoretic properties compared with those of casein that had not been complexed.

The uncomplexed casein was obtained by centrifuging unheated skimmilk at 105,000 × g for 90 min. The ultracentrifuge patterns of the respective protein systems dissolved in veronal buffer, pH 8.4, 0.1 ionic strength, are shown in Figure 3. The photographs were all taken at 102 to 104 min. after sedimentation began. The temperature of centrifugation was from 20 to 27° C.

Figure 3-A is the ultracentrifuge pattern of the casein obtained from skimmilk, as described above.

Figure 3-B is the protein system obtained by treating the complex with E.D.T.A. or oxalate ion as ammonium oxalate, followed by centrifugation to remove the fat and calcium oxalate. The solution was then dialyzed against several changes of buffer to remove excess oxalate ion. Except for better resolution of sedimenting components in B, the patterns of B and A are essentially the same.

Figure 3-C is the ultracentrifuge pattern of the protein system of the complex recovered after disintegration in *n*-butanol. This treatment appears to have shifted the bulk of the faster-sedimenting components to that of the component marked 1.

Figure 3-D is the ultracentrifuge pattern of the protein system recovered from the complex by extracting it three times with ethyl ether and ethanol (3:1-v/v). This treatment destabilizes most of the faster-sedimenting material. The latter sedimented rapidly at the beginning of centrifugation and formed a clear gel in the bottom of the centrifuge cell. It could not be redispersed as a stable solution in veronal buffer.

Although the protein system in the complex is predominantly casein, the manner in which it is extracted from the complex alters either the number of sedimenting boundaries or the distribution of material in the sedimenting boundaries.

Mode of interaction. To determine how the fat is complexed with the calcium caseinate, the following experiments were performed:

a. Butter oil was homogenized into milk dialysate at 8,000 p.s.i. The dialysate was prepared by dialyzing distilled water against a large volume of milk. This homogenate was then heated to 145° F. and mixed at a concentration of 3.3% with skimmilk (also heated to 145° F.) and with skimmilk which had been homogenized at 8,000 p.s.i. In neither experiment was a sedimentable

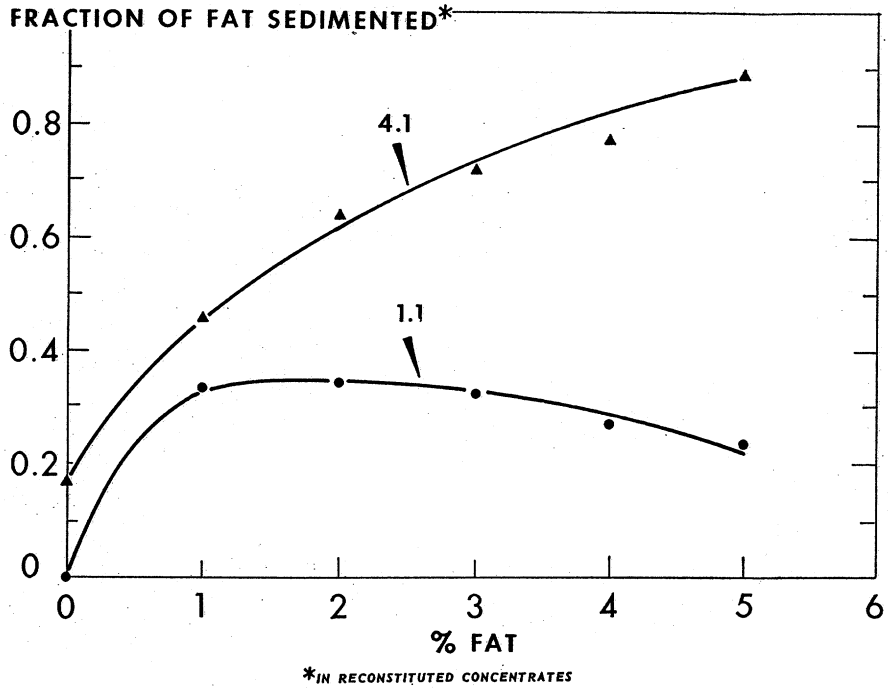

FIG. 4. Fraction of fat sedimented in milks and concentrated milks containing different fat contents. Homogenization pressure, 8,000 p.s.i.

fat-protein complex formed. This experiment shows that neither fresh fat surfaces nor the fineness of dispersion are sufficient to cause the fat to enter the protein phase.

b. Butter oil was homogenized directly into skimmilk at a concentration of 3.3%. The fat-protein complex was formed. In conjunction with the previous experiments, this shows that the interaction depends upon the conditions which exist at the homogenizer valve at the instant of homogenizing. It further illustrates that the fat globule membrane is not necessary for the formation of the complex.

c. Mineral oil was homogenized into skimmilk at a concentration of 3.3%. The fat-protein complex was formed. Since mineral oil does not contain any reactive or polar groups, the binding force must be by van der Waals forces between hydrocarbon sites on the caseinate complex and the fat. Because such binding occurs only during homogenizing, homogenizing must distort the caseinate micelle and expose hydrocarbon sites not otherwise available. The extent of such distortion and, therefore, the amount of fat bound would depend upon the homogenizing pressure.

Complex formation as a function of fat content. The previous experiments were all conducted on milk containing 12.4% T.S. and 3.3% fat—or some concentration of this milk. The effect of different fat content on complex formation was determined by the following experiment. A single lot of milk was separated and the cream diluted to 10% fat with the skimmilk. One part of the skimmilk was divided into six samples and sufficient 10% milk added to each sample to give a range of fat content of from 0 to 5%. The second lot of the skimmilk and the remaining 10% milk were concentrated to 32% solids-not-fat.

The concentrated skimmilk was divided into six lots and sufficient concentrated 10% milk (now concentrated to 39% fat) was added to the different samples to give a range of fat content of 0 to 20% fat. The samples were heated to 145° F. and homogenized at 8,000 p.s.i. The results are shown in Figure 4. In nonconcentrated milk the amount of fat complexed at a given solids-not-fat level reaches a limiting value, but in the 4:1 concentrated milk a maximum value for the formation of the complex was not reached in the range of fat content examined. There is either a factor limiting complex formation at a 1:1 concentration or a factor facilitating complex formation at a 4:1 concentration. This factor must be associated with the solids-not-fat content rather than the fat content, since in the 1:1 concentration a limiting value for complex formation is reached which does not occur at the 4:1 concentration.

In Figure 5 the fraction of the fat complex per gram of sedimenting nitrogen is plotted as a function of the free fat. By free fat is meant that fat which is not sedimented. In the accepted sense the free ligand concentration is an equilibrium concentration, i.e., it exerts a pressure on the bound ligand to maintain a constant distribution of ligand between free and bound states. The free fat plotted in Figure 5 is not an equilibrium fat at the time of analysis, but may have been such at the instant of homogenization. Assuming that it

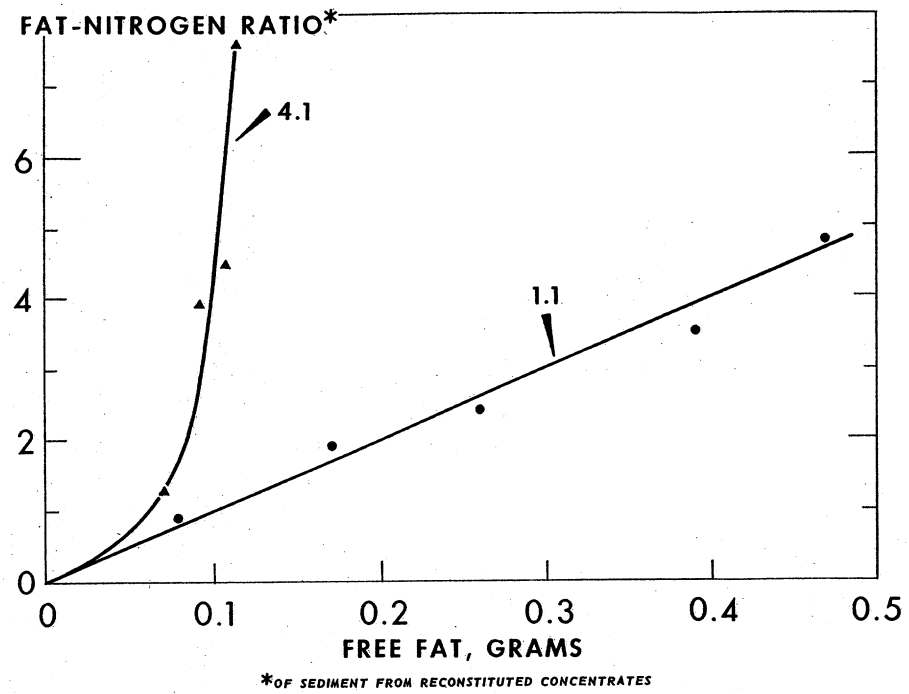

FIG. 5. The binding of fat by nonconcentrated and concentrated milks homogenized at 8,000 p.s.i.

does represent an equilibrium fat, the data of Figure 5 indicate that at a 1:1 concentration the fat partitions between the complex and the fat phase. This represents a statistical type of binding in which the amount of fat bound is proportional to the amount already bound, i.e., to the number of binding sites available. Since casein is the binding moiety a different interpretation may be made of the statistical concept. The distribution of sizes of caseinate micelles may be substituted for the distribution of the number of binding sites available. Whether this size distribution is continuous, as reported by Nichols *et al.* (5), or discrete, as reported by Ford and Ramsdall (3), the binding curve would in either case illustrate a partition effect when the size of the caseinate micelles is the only interaction parameter.

The curve for the fat complexed at a 4:1 concentration illustrates a cooperative kind of binding, in which the more fat that is bound, the easier it is for additional fat to be bound.

Another difference in the method of complexing of fat by 1:1 milk as compared with a 4:1 concentrate of the same milk is illustrated in Figure 6. To obtain the data of Figure 6, sucrose in increasing concentrations was added to aliquots of the 1:1 milk and to aliquots of the reconstituted concentrate. These solutions were centrifuged at increasing times proportional to their increase in viscosity; fat and nitrogen analyses were made of the sediments and the specific gravity of the supernatant liquids determined. The sedimenting complex of the reconstituted 4:1 concentrate underwent flotation at a specific

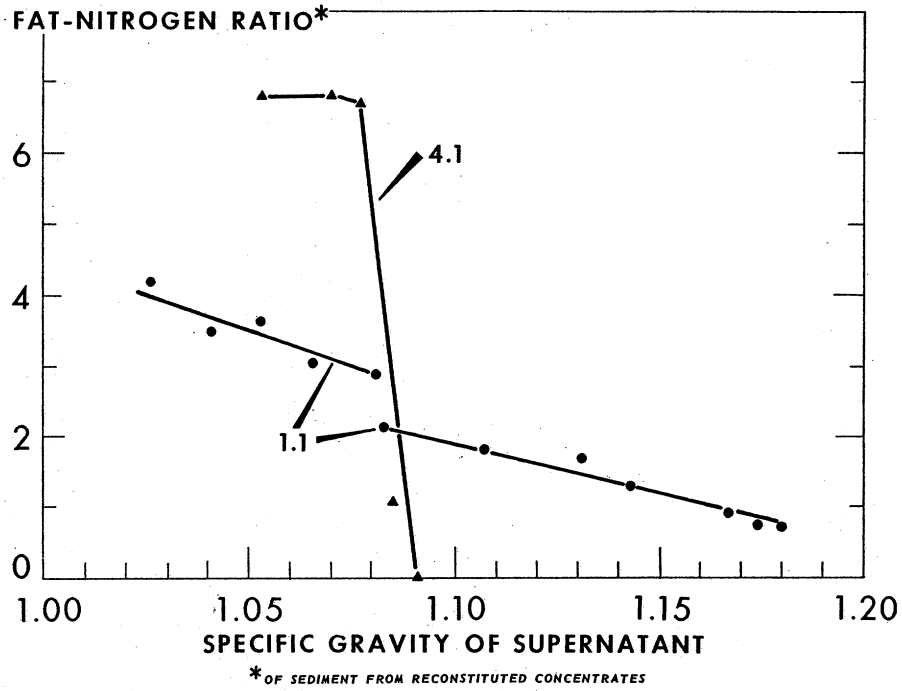

FIG. 6. Effect of concentration on specific gravity of fat-protein complex formed by homogenization. Homogenization pressure, 8,000 p.s.i. The 4:1 concentrate was reconstituted to 12.4% T.S. before adjustment of specific gravity with sucrose.

gravity of between 1.077 and 1.085 (20° C.). This narrow range of specific gravity of the complex indicates that only one species of complex forms when a 4:1 concentrate is homogenized at 8,000 p.s.i. In contrast, the fat-protein complexes formed in 1:1 milk homogenized at 8,000 p.s.i. have a range of different F/N ratios.

Effect of calcium on the formation of the fat-protein complex. It is usually necessary to consider the behavior of casein in terms of the amount of calcium in the system. To determine the effect of calcium on the formation of the fat-protein complex, different amounts of calcium as $CaCl_2 \cdot 2H_2O$ were added to aliquots of pasteurized milk (145° F. for 30 min.) and the milk allowed to equilibrate for 24 hr. at 36° F. The different samples of milk were warmed to 110° F. and homogenized at 8,000 p.s.i. The results are presented graphically in Figure 7. The addition of as little as 2.5 m*M* of calcium ions causes an increase in the amount of fat complexed; between 10 and 20 m*M* of calcium is sufficient to complex 100% of the fat. When the specific gravity of the solution containing 10 m*M* of added calcium was increased to 1.07 the fat-protein complex abruptly underwent flotation. The amount of fat complexed and the limited specific gravity range of the complex formed indicate that calcium causes formation of a fat-protein complex similar to that formed in a 4:1 concentrated milk.

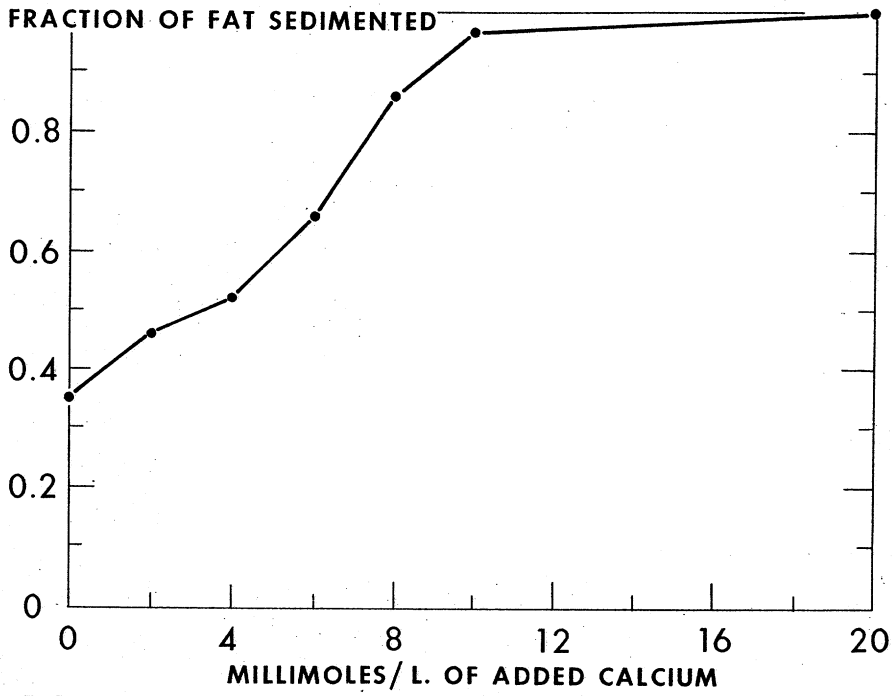

FIG. 7. Effect of increasing calcium ion concentration on formation of fat-protein complex.

DISCUSSION

It has been demonstrated that milk fat and the caseinate micelles of milk interact when homogenized. From the data obtained, certain speculations may be made about the mode of interaction. These speculations concern the effect of homogenizing upon the caseinate micelles, the effect of concentration upon the caseinate micelles, and the structure of the fat-caseinate complex.

The fat-protein interaction could not be induced by separately homogenizing the skimmilk and the fat and then mixing them. The chemistry of fat suggests that the only effect of homogenization on it should be increased surface area and, thereby, increased surface energy. If increase in surface energy or decrease in size were the only requirements for the interaction, it should occur when the fat homogenate is mixed with the skimmilk. That an interaction does not occur except at the homogenizing valve must mean that caseinate micelles are, in some manner, activated for the interaction by homogenizing.

In another experiment, mineral oil was complexed with the caseinate micelles by homogenization. Hydrocarbons should adsorb to the caseinate micelles only on a hydrocarbon site. Such hydrophobic sites, to be consistent with the accepted structure of proteins in aqueous solution, would be within the micelles. Therefore, homogenizing must distort or open up the caseinate micelle. If the hydrocarbon portion were on the surface of the micelle, the amount of fat bound would be proportional to the micelle surface area. Assuming a sphere,

the fat bound would be proportional to the square of the micelle diameter, or L^2. The nitrogen content would be proportional to the volume, or L^3. The fat-nitrogen ratio would be L^2/L^3 (=1/L) or, the smaller the micelle, the more fat would be complexed to a given amount of casein. But, in the experiment in which calcium was added to milk to determine its effect on complex formation, the amount of fat complexing (and the F/N) increased with micelle size, and not the converse. Therefore, the part of the micelle to which the fat is bound must not be on its surface.

A construct which describes the increase in F/N ratio with increase in micelle size is one having the caseinate micelle distorted into a shell about the fat. In this case, the fat complexed is proportional to the volume of the shell and the nitrogen is proportional to its area, or L^2. The F/N ratio becomes L^3/L^2 (=L) and increases with micelle size. A variation in the F/N ratio would also be consistent with the data for the F/N ratio in Figure 6, since a distribution in size of caseinate micelles occurs in normal milk (3, 5).

The extent of fat-protein interaction in 4:1 concentrated milk, as compared with that in the same milk not concentrated, implies that concentration causes changes in the micelle structure. The concentration of milk fat produces no known changes in its physical and chemical properties. The ratio of total solids to fat is the same in the 4:1 concentrate as in the 1:1 milk. If the increased interaction between fat and casein at a 4:1 concentration resulted from closer proximity of the fat and casein during homogenization, the interaction should increase linearly with concentration. This does not occur. Instead, the concentration effect is critical in behavior, which implies a change in the physical and/or chemical properties of the caseinate micelle. This change is manifested in two ways: (1) a large increase in the amount of fat bound, and (2) the fat-protein complex formed becomes essentially of one discrete size. If the interpretation is correct, that the amount of fat bound is a function of micelle size, only one size of caseinate micelle exists at a concentration of 37% T.S. and above.

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